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# Event-specific Method for the Quantification of Soybean MON87769 Using Real-time PCR

## Validation Report and Validated Method

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# Validation Report

**17 January 2012**

**Joint Research Centre**
**Institute for Health and Consumer Protection**
**Molecular Biology and Genomics Unit**

## Executive Summary

The European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the MON87769 transformation event in soybean DNA (unique identifier MON-87769-7). The collaborative trial was conducted according to internationally accepted guidelines [1, 2].

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed" and to Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003", Monsanto provided the detection method and the control samples (genomic DNA extracted from soybean seeds harbouring the MON87769 event and from conventional soybean seeds). The EU-RL GMFF prepared the validation samples (calibration samples and samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from nine European countries.

The results of the international collaborative trial met the ENGL performance requirements. The method is therefore considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Regulation (EC) No 641/2004.

The results of the collaborative study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

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# Report on Steps 1-3 of the Validation Process

Monsanto submitted the detection method and control samples for soybean event MON 87769 (unique identifier MON-87769-7) according to Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the EU-RL GMFF Validation Process", http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories (ENGL) and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, the scientific assessment was performed for soybean event MON87769 and positively concluded in February 2010.

Between October 2009 and June 2010, the EU-RL GMFF verified the purity of the control samples provided, and requests for replacement of samples were made. Upon reception of additional samples, the method characteristics were verified by quantifying five GM levels within the range 0.1%-9.0% on a copy number basis (step 3, experimental testing of samples and methods). The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision of the quantifications were within the limits established by the ENGL. The DNA extraction module of the method was previously tested on samples of food and feed and a report was published on the EU-RL GMFF website on the 19th of September 2007 (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

A Technical Report summarising the results of tests carried out by the EU-RL GMFF (step 3) is available on request.

# Content

# 1. Introduction

Monsanto provided the detection method and control samples for soybean event MON87769 (unique identifier MON-87769-7) according to Articles 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The European Union Reference Laboratory for Genetically Modified Food and Feed, established by Regulation (EC) No 1829/2003, organised the international collaborative study for the validation of the event-specific method for the detection and quantification of soybean event MON87769. The study involved twelve laboratories, among those listed in Annex II ("National reference laboratories assisting the EURL for testing and validation of methods for detection") of Regulation (EC) No 1981/2006 of 22 December 2006.

Upon reception of method, samples and related data (step 1), the EU-RL GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the method (step 3) according to the requirements of Commission Regulation (EC) No 641/2004. The internal experimental evaluation of the method was carried out between October 2009 and June 2010.

Following the evaluation of the data and the results of the internal laboratory tests, the international collaborative study was organised (step 4) and took place in July 2010.

A method for DNA extraction from seeds and grains followed by PEG precipitation, submitted by the applicant, was evaluated by the EU-RL GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on method testing are available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm

The collaborative study aimed at validating a quantitative real-time PCR (Polymerase Chain Reaction) method. The method is an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of event MON87769 DNA to total soybean DNA. The procedure is a simplex system, in which the soybean *Le1* (lectin) endogenous assay and the target assay (MON87769) are performed in separate wells.

The international collaborative study was carried out in accordance with the following internationally accepted guidelines:

- ISO 5725 (1994) [(1)]
- The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995) [(2)]

# 2. Selection of participating laboratories

As part of the international collaborative study the method was tested in twelve laboratories to determine its performance.

In July 2010, the EU-RL GMFF invited all National Reference Laboratories nominated under Regulation (EC) No 1981/2006 of 22 December 2006 to participate in the validation study of the quantitative real-time PCR method for the detection and quantification of soybean event MON87769. The National Reference Laboratories are listed in the Annex II ("National reference laboratories assisting the EURL for testing and validation of methods for detection") of the same Regulation.

Thirty-six laboratories expressed in writing their willingness to participate, two declined the invitation, while thirty-one did not answer. The EU-RL GMFF performed a random selection of twelve laboratories out of those that responded positively to the invitation, making use of a validated software application.

Clear guidance was given to the selected laboratories with regards to the standard operational procedures to follow for the execution of the protocol. The participating laboratories are listed in Table 1.

Table 1. Laboratories participating in the validation of the detection method for soybean MON 87769.

| Laboratory | Country |
|---|---|
| Laboratory for the Detection of GMO in Food | DE |
| Center for Agricultural Technology Augustenberg | DE |
| RIKILT Institute of Food Safety | NL |
| National Health Laboratory, Food Control Department | LU |
| Genetically Modified Organism Controlling Laboratory | PL |
| Institute for Agricultural and Fisheries Research (ILVO) | BE |
| National Veterinary Research Institute in Pulawy, Department of Feed Hygiene | PL |
| Italian National Institute for Health - Department of Veterinary Public Health and Food Safety - Unit GMOs and Mycotoxins | IT |
| Scientific Institute of Public Health (IPH) | BE |
| National Food Institute, Dept. of Toxicology and Risk Assessment | DK |
| BioGEVES - Groupement d'Intérêt Public – Groupe d'Etude et de contrôle des Variétés et des Semences | FR |
| National Food and Veterinary Risk Assessment Institute, Laboratory Department, Molecular Biology and GMO Section | LT |

# 3. Materials

For the validation of the quantitative event-specific method, control samples consisted of:

*i)* genomic DNA extracted from homozygous soybean seeds harbouring the event MON87769 (ID: MON87769-20090727), and
*ii)* genomic DNA extracted from conventional soybean seeds (ID: C.SOY-20090727).

Samples were provided by the applicant in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 [control sample defined as "the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)"].

Samples containing genomic DNA mixtures of soybean MON87769 and non-GM soybean at different GMO contents were prepared by the EU-RL GMFF, using the control samples provided, in a constant amount of total soybean DNA.

Participants received the following materials:

- ✓ Five calibration samples (150 µL of DNA solution each) labelled from S1 to S5.
- ✓ Twenty unknown DNA samples (75 µL of DNA solution each) labelled from U1 to U20.
- ✓ Reaction reagents as follows:
  - universal PCR Master Mix (2x), four bottles: 5 mL each
  - distilled sterile water, one tube: 25 mL

- ✓ Primers and probes (1 tube each) as follows:

  *Le1* reference system

  | | | |
  |---|---|---|
  | ▪ *Le1* primer 1 | (10 µM): | 250 µL |
  | ▪ *Le1* primer 2 | (10 µM): | 250 µL |
  | ▪ *Le1* probe | (5 µM): | 180 µL |

  MON87769 system

  | | | |
  |---|---|---|
  | ▪ MON 87769 forward primer | (10 µM): | 1000 µL |
  | ▪ MON 87769 reverse priemr | (10 µM): | 1000 µL |
  | ▪ MON 87769 probe | (5 µM): | 720 µL |

# 4. Experimental design

Twenty unknown samples (labelled from U1 to U20), representing five GM (%) contents, were used in the validation study (Table 2). On each PCR plate, the samples were analysed for the MON87769 specific system and for the *Le1* reference system. Two plates were run per participating laboratory with two replicates for each GM level. In total, four replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. Participating laboratories carried out the determination of the GM% according to the instructions provided in the protocol and using the electronic tool provided.

Table 2. MO 87769 GM (%) contents

| MON 87769 GM% [GM copy number/maize genome copy number (x 100)] |
|---|
| 0.1 |
| 0.5 |
| 0.9 |
| 5.0 |
| 9.0 |

# 5. Method

For the detection of MON87769 genomic DNA, an 87 bp fragment of the region spanning the 3' plant-to-insert junction in soybean MON87769 event is amplified using specific primers. PCR products are measured at each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM as a reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For the relative quantification of event MON87769, a soybean-specific reference system amplifies a 74 bp fragment of *Le1*, a soybean endogenous gene, using *Le1* gene-specific primers and a *Le1* specific probe labelled with FAM as a reporter dye and TAMRA as a quencher dye.

Standard curves are generated for both the MON87769 and the *Le1* specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the copy numbers in the unknown sample DNA are estimated by interpolation from the standard curves.

For relative quantification of event MON87769 DNA in a test sample, the MON 87769 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 to obtain the percentage value (GM% = MON87769 /*Le1* x 100).

Calibration sample S1 was prepared by mixing the appropriate amount of MON87769 DNA in control non-GM soybean DNA to obtain a 10% GM MON87769. Sample S2 was prepared by fourfold dilution from the S1 sample as presented in Table 3.

The absolute copy numbers of the calibration curve samples are determined by dividing the DNA mass (nanograms) by the published average 1C value for soybean genome (1.13 pg) [(3)]. The copy number values used in the quantification, the GM contents of the calibration samples and the total DNA quantity used in the PCR reactions are provided in Table 3.

Table 3. Copy number values of the standard curve samples.

| Sample code | S1 | S2 | S3 | S4 | S5 |
|---|---|---|---|---|---|
| Total amount of DNA in reaction (ng) | 200 | 50 | 10 | 5 | 1.67 |
| Soybean genome copies | 176991 | 44248 | 8850 | 4425 | 1475 |
| MON 87769 GM soybean copies | 17699 | 4425 | 885 | 442 | 147 |

# 6. Deviations reported

Eleven laboratories reported no deviations from the protocol.

One laboratory reported the use of a reaction volume of 25 µL.
One laboratory erroneously copied the Ct values for the event-specific method into the cells allocated to the taxon-specific reference system for the samples of the standard curve in plate B. The EU-RL GMFF corrected such mistake during data analysis

# 7. Results

### *PCR efficiency and linearity*

The values of the slopes [from which the PCR efficiency is calculated using the formula ((10^(-1/slope))-1) x 100) of the standard curve and of the $R^2$ (indicating the linearity of the regression) reported by participating laboratories for the MON87769 system and the *Le1* reference system are summarised in Table 4.

The mean PCR efficiency was 97% for the MON87769 assay and 100% for the *Le1* assay, with both values within the ENGL acceptance criteria. The linearity of the method was 1.00 for both the MON87769 and *Le1* assays.

These results confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

Table 4. Values of slope, PCR efficiency and $R^2$

| | | **MON 87769** | | | ***Le1*** | | |
|---|---|---|---|---|---|---|---|
| **Lab** | **Plate** | **Slope** | **PCR Efficiency (%)** | **$R^2$** | **Slope** | **PCR Efficiency (%)** | **$R^2$** |
| 1 | A | -3.41 | 97 | 0.99 | -3.38 | 98 | 1.00 |
| | B | -3.29 | 101 | 1.00 | -3.31 | 100 | 1.00 |
| 2 | A | -3.35 | 99 | 1.00 | -3.29 | 101 | 1.00 |
| | B | -3.32 | 100 | 1.00 | -3.27 | 102 | 1.00 |
| 3 | A | -3.43 | 96 | 1.00 | -3.41 | 97 | 1.00 |
| | B | -3.32 | 100 | 1.00 | -3.38 | 98 | 1.00 |
| 4 | A | -3.43 | 96 | 1.00 | -3.40 | 97 | 1.00 |
| | B | -3.51 | 93 | 0.99 | -3.38 | 98 | 1.00 |
| 5 | A | -3.38 | 98 | 1.00 | -3.22 | 105 | 1.00 |
| | B | -3.38 | 98 | 1.00 | -3.33 | 100 | 1.00 |
| 6 | A | -3.50 | 93 | 1.00 | -3.32 | 100 | 1.00 |
| | B | -3.46 | 94 | 1.00 | -3.36 | 98 | 1.00 |
| 7 | A | -3.56 | 91 | 0.98 | -3.35 | 99 | 0.99 |
| | B | -3.63 | 89 | 0.97 | -3.30 | 101 | 1.00 |
| 8 | A | -3.31 | 101 | 1.00 | -3.34 | 99 | 1.00 |
| | B | -3.41 | 96 | 1.00 | -3.37 | 98 | 1.00 |
| 9 | A | -3.33 | 100 | 1.00 | -3.35 | 99 | 1.00 |
| | B | -3.34 | 99 | 1.00 | -3.40 | 97 | 1.00 |
| 10 | A | -3.42 | 96 | 1.00 | -3.35 | 99 | 0.98 |
| | B | -3.29 | 101 | 1.00 | -3.31 | 101 | 1.00 |
| 11 | A | -3.44 | 95 | 1.00 | -3.29 | 101 | 1.00 |
| | B | -3.36 | 98 | 1.00 | -3.32 | 100 | 1.00 |
| 12 | A | -3.35 | 99 | 1.00 | -3.22 | 104 | 1.00 |
| | B | -3.35 | 99 | 1.00 | -3.30 | 101 | 1.00 |
| | Mean | -3.40 | 97 | 1.00 | -3.33 | 100 | 1.00 |

## *GMO quantification*

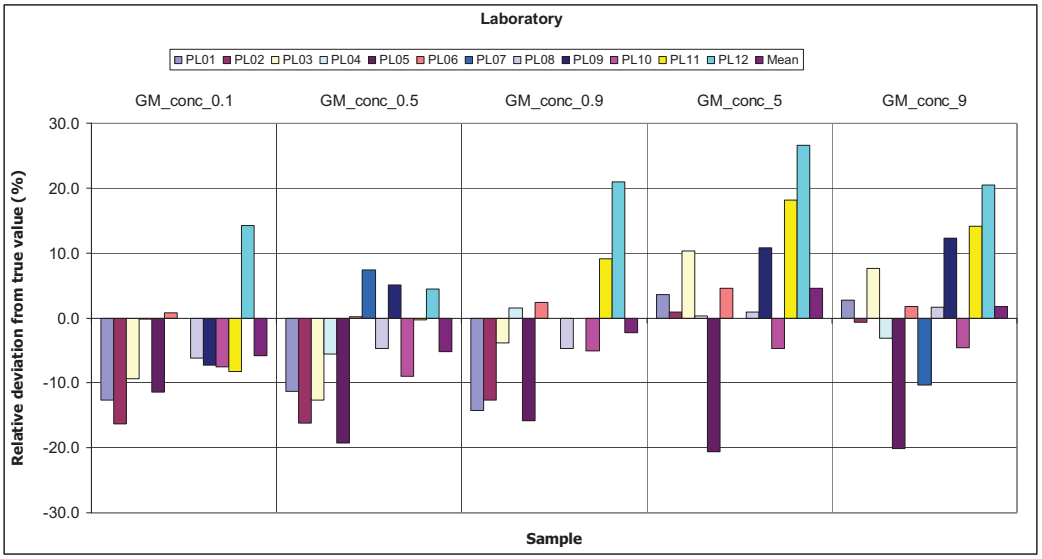
Table 5 summarises the mean GM% values of the four replicates for each GM level as provided by all laboratories. Each mean value is the average of three PCR repetitions.

Table 5. GMO% mean values determined by laboratories for unknown samples.

| | GMO content (GMO% = GMO copy number/soybean genome copy number x 100) | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LAB | 0.1 | | | | 0.5 | | | | 0.9 | | | | 5.0 | | | | 9.0 | | | |
| | REP 1 | REP 2 | REP 3 | REP 4 | REP 1 | REP 2 | REP 3 | REP 4 | REP 1 | REP 2 | REP 3 | REP 4 | REP 1 | REP 2 | REP 3 | REP 4 | REP 1 | REP 2 | REP 3 | REP 4 |
| 1 | 0.11 | 0.08 | 0.07 | 0.09 | 0.38 | 0.37 | 0.49 | 0.53 | 0.78 | 0.75 | 0.78 | 0.78 | 5.48 | 4.91 | 5.45 | 4.87 | 8.62 | 9.01 | 9.65 | 9.69 |
| 2 | 0.09 | 0.09 | 0.08 | 0.07 | 0.39 | 0.41 | 0.46 | 0.41 | 0.79 | 0.81 | 0.82 | 0.72 | 4.90 | 5.05 | 5.17 | 5.06 | 9.03 | 8.41 | 9.18 | 9.13 |
| 3 | 0.10 | 0.09 | 0.10 | 0.07 | 0.37 | 0.40 | 0.49 | 0.49 | 0.77 | 1.11 | 0.80 | 0.78 | 5.56 | 5.35 | 5.43 | 5.72 | 10.17 | 9.10 | 9.85 | 9.61 |
| 4 | 0.11 | 0.09 | 0.10 | 0.10 | 0.42 | 0.47 | 0.47 | 0.53 | 0.94 | 0.87 | 0.96 | 0.89 | 4.98 | 4.77 | 5.13 | 5.19 | 9.18 | 8.35 | 8.76 | 8.59 |
| 5 | 0.09 | 0.09 | 0.10 | 0.08 | 0.47 | 0.38 | 0.43 | 0.33 | 0.89 | 0.75 | 0.75 | 0.65 | 4.53 | 3.72 | 3.66 | 3.98 | 6.09 | 7.34 | 8.50 | 6.85 |
| 6 | 0.10 | 0.11 | 0.11 | 0.09 | 0.46 | 0.52 | 0.47 | 0.55 | 0.96 | 0.94 | 0.88 | 0.91 | 4.92 | 6.13 | 4.82 | 5.05 | 9.57 | 8.44 | 8.42 | 10.22 |
| 7 | 0.16 | 0.13 | 0.06 | 0.10 | 0.43 | 0.57 | 0.67 | 0.49 | 0.84 | 1.03 | 1.32 | 0.36 | 3.79 | 4.96 | 6.10 | 4.02 | 10.15 | 6.68 | 10.30 | 5.15 |
| 8 | 0.10 | 0.01 | 0.01 | 0.07 | 0.43 | 0.42 | 0.48 | 0.58 | 0.90 | 0.91 | 0.84 | 0.78 | 5.34 | 4.24 | 5.43 | 5.17 | 10.67 | 8.91 | 9.38 | 7.62 |
| 9 | 0.08 | 0.09 | 0.11 | 0.08 | 0.58 | 0.57 | 0.51 | 0.45 | 1.00 | 1.38 | 0.92 | 0.80 | 5.61 | 5.69 | 4.85 | 6.02 | 8.96 | 11.89 | 9.87 | 9.73 |
| 10 | 0.09 | 0.10 | 0.10 | 0.09 | 0.43 | 0.42 | 0.36 | 0.47 | 0.85 | 0.93 | 0.63 | 0.78 | 5.25 | 5.25 | 4.35 | 4.42 | 8.35 | 9.36 | 8.01 | 9.37 |
| 11 | 0.12 | 0.07 | 0.07 | 0.10 | 0.47 | 0.48 | 0.48 | 0.57 | 1.05 | 1.04 | 0.99 | 0.84 | 5.83 | 6.58 | 5.59 | 5.63 | 10.51 | 11.03 | 8.53 | 11.02 |
| 12 | 0.11 | 0.13 | 0.10 | 0.11 | 0.50 | 0.50 | 0.41 | 0.68 | 1.23 | 1.17 | 0.94 | 1.02 | 6.32 | 6.27 | 6.36 | 6.38 | 13.43 | 8.78 | 9.12 | 12.03 |

In Figure 1, the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the relative GM quantification obtained by the participating laboratories. The purple bar at the far right of each panel represents the overall mean value for each GM level.

Figure 1. Relative deviation (%) from the true value of MON87769 for all laboratories




The mean relative deviations from the true values are negative for GM levels 0.1%, 0.5% and 0.9% and positive for the upper part of the dynamic range. However, on average, the relative deviations from the true values are below 10% over the whole dynamic range. Overall, the average relative deviation is within the acceptance criterion at all GM levels tested indicating a satisfactory trueness of the method.

# 8. Method performance requirements

Among the performance criteria established by ENGL and adopted by the EU-RL GMFF (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm, see also Annex 1), repeatability and reproducibility are assessed through an international collaborative trial, carried out with the support of twelve ENGL laboratories (see Table 1).

Table 6 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the collaborative trial. The relative reproducibility standard deviation ($RSD_R$), describing the inter-laboratory variation, should be below 33% at the target concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 6, the method satisfies this requirement at all GM levels tested. In fact, the highest value of $RSD_R$ is 16% at the 9.0% GM level, thus well within the acceptance criterion.

Table 6. MON 87769: summary of validation results.

| | Expected value (GMO%) | | | | |
|---|---|---|---|---|---|
| **unknown sample GMO %** | **0.1** | **0.5** | **0.9** | **5.0** | **9.0** |
| Laboratories having returned valid results | 12 | 12 | 12 | 12 | 12 |
| Samples per laboratory | 4 | 4 | 4 | 4 | 4 |
| Number of outliers | 1 | 0 | 2 | 1 | 0 |
| Reason for exclusion | 1 C | - | 2 C | 1 C | - |
| Mean value | 0.09 | 0.47 | 0.88 | 5.2 | 9.2 |
| Relative repeatability standard deviation, $RSD_r$ (%) | 13 | 14 | 9.7 | 7.0 | 14 |
| Repeatability standard deviation | 0.01 | 0.07 | 0.09 | 0.37 | 1.25 |
| Relative reproducibility standard deviation, $RSD_R$ (%) | 15 | 15 | 14 | 13 | 16 |
| Reproducibility standard deviation | 0.01 | 0.07 | 0.13 | 0.69 | 1.47 |
| Bias (absolute value) | -0.01 | -0.03 | -0.02 | 0.23 | 0.16 |
| Bias (%) | -5.8 | -5.2 | -2.2 | 4.6 | 1.8 |

C = Cochran's test; G = Grubbs' test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2. Bias is estimated according to ISO 5725 data analysis protocol.

Table 6 further documents the relative repeatability standard deviation ($RSD_r$), as estimated for each GM level. In order to accept methods for collaborative study evaluation, the EU-RL GMFF requires that the $RSD_r$ value is below 25%, as indicated by the ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). As it can be observed from the values reported, the method showed a repeatability standard deviation below 25% at all GM levels, with the highest value of $RSD_r$ of 14% at 0.5 % and 9% GM levels.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to the ENGL method performance requirements, trueness should be ± 25% across the entire dynamic range. In this case, the method satisfies this requirement across the dynamic range tested; in fact the highest value of bias is – 5.8% at the 0.1% GM level.

# 9. Conclusions

The overall method performance has been evaluated with respect to the method acceptance criteria and method performance requirements recommended by the ENGL (as detailed at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm). The method acceptance criteria were reported by the applicant and used to evaluate the method prior to the international collaborative study (see Annex 1 for a summary of method acceptance criteria and method performance requirements).

The results obtained during the collaborative study indicate that the analytical module of the method submitted by the applicant complies with ENGL performance criteria.

Therefore, the method is considered applicable to the control samples provided (see paragraph 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

# 10. Quality assurance

The EU-RL GMFF operates according to ISO 9001:2008 (certificate number: CH-32232) and technical activities under ISO 17025:2005 [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative / quantitative PCR) – Accredited tests available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7]

# 11. References

1. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
2. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem.* 67, 331-343.
3. Royal Botanic Garden, Kew. Plant DNA c-values database. http://data.kew.org/cvalues/

# 12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

**Method Acceptance Criteria**

***Applicability***

Definition: The description of analytes, matrices and concentrations to which the method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

***Practicability***

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. cost/sample) of the method.

Acceptance criteria: The method should generally be practicable in line with other methods for a similar purpose. More specifically the method is deemed unacceptable, unless suitable justification is supplied, if:

- it requires a new type of apparatus (not generally available) or expensive equipment; or
- the resources required to perform the method (time, workload, reagents, costs) are considerably higher than the resources required to perform other methods for similar purpose.

Other practicability considerations may also deem the method impracticable.

***DNA Extraction and Purification***

The aim of a DNA extraction procedure is to provide DNA of suitable quality for subsequent analysis. DNA quality depends on the average length, structural integrity and chemical purity of the extracted DNA.
It is recognised that highly fragmented DNA and co-extracted impurities of a DNA preparation may hinder the correct process of detecting and quantifying genetically modified DNA. Food and feed made

of various ingredients may exert a matrix effect, depending on the DNA extraction method employed, and impair the sensitivity of the following analytical approach. For this purpose, critical steps of DNA extraction and purification should be clearly highlighted in the technical documentation accompanying a method and acceptance criteria are established to allow objective determination of PCR quality of DNA extracts which can be considered suitable for subsequent detection experiments (e.g. qualitative and/or quantitative PCR).
DNA extraction procedures should result in repeatable recovery, fragmentation profile, concentration and PCR quality of DNA extracts. As such, it is recommended to process the given DNA extraction protocol on different days (e.g. 3 days) with an adequate number of test portions (e.g. 6 per day).
In agreement with international guidelines (e.g. EN ISO 21571[Error! Reference source not found.], EN ISO 24276[Error! Reference source not found.]) the following criteria are used to assess method performance.

*a) DNA concentration*

Definition: amount of an analyte per unit volume of solution

Acceptance criterion: The DNA extraction method employed shall be appropriate to obtain the quantity of nucleic acid required for the subsequent analysis. The DNA concentration measured as weight of the analyte/volume of solution should be higher than the working concentration described in the protocol of the detection method.
Example: if the RT-PCR protocol indicates 40 ng/µL as the DNA concentration of the DNA solution to be added to the master-mix, the concentration of the DNA extract should be > 40 ng/µL.

*b) DNA fragmentation state*

Definition: Breakage of genomic (high molecular weight) DNA into smaller DNA fragments

Acceptance criterion: For quantitative (real time-based) analysis, the molecular weight of the extracted DNA sample should be at least higher than the amplicon size produced by the event specific and the taxon specific reference systems as established by comparison with a reference nucleic acid marker.
For qualitative analysis, in case of DNA suspensions to be used in qualitative analysis, the presence of a certain proportion of DNA molecules of molecular weight lower than the amplicon size produced by the method may be considered acceptable.

*c) Purity of DNA extracts*

Definition: the absence of co-extracted compounds in a DNA sample impairing the efficiency of the PCR reactions and leading to a delay in the onset of the exponential phase of the amplification profile

Acceptance criterion: The difference (ΔCt) average between the measured Ct value and the extrapolated Ct value of the first diluted sample of the inhibition test should be <0.5. [(measured Ct – extrapolated Ct)] <0.5 and the slope of the inhibition curve should be within -3.6 and -3.1.
The preferred PCR assay for the inhibition test is the internal control assay (e.g. the taxon specific reference system). The total DNA amount in the first sample of the dilution series should be not less than the total DNA amount used in the submitted method (e.g. the DNA amount indicated in the PCR protocol of the taxon specific reference system).

***Specificity***

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should not produce amplification signals with target sequences different for the target sequence for which the method was developed. This should be demonstrated by similarity searches against databases (e.g. EMBL, GenBank, Patent, etc.) and with empirical results from testing the method with non-target transgenic events and non-transgenic material.
For detection of specific GM events, the target sequence shall be event specific.
For taxon specific target sequences (target sequence), the absence of allelic and copy-number variation across a globally representative and diverse sample of the species variety shall be demonstrated. Allelic and/or copy-number variation in other lines shall be reported if such variation is known by the applicant.
The specificity of the target sequence shall be verified by *in silico* studies against publicly available sequence databases (e.g. EMBL, GenBank, etc.) and experimentally by demonstrating the absence of amplification products when the target sequence specific assay is applied to individual PCRs of pure genomic DNA of a representative sample of the closest relatives to the target taxa as well as of the most important food crops.

***Dynamic Range***

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of trueness and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The range of the standard curve(s) for real-time PCR should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) ends.
Example: 0.09% and 4.5% for a 0.9% GMO concentration or 50 and 2500 genome copies if the target is 500 copies.

***Trueness***

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness shall be within ±25% of the accepted reference value over the whole dynamic range.

***Amplification Efficiency***

Definition: The rate of amplification that leads to a theoretical slope of –3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation:

$$Efficiency = 10^{\left(\frac{-1}{slope}\right)} - 1$$

Acceptance Criterion: The average value of the slope of the standard curve shall be in the range of (-3.1 ≥ slope ≥ -3.6)

***$R^2$ Coefficient***

Definition: The $R^2$ coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of $R^2$ shall be ≥0.98.

***Precision - Relative Repeatability Standard Deviation (RSDr)***

Definition: The relative standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be ≤25% over the whole dynamic range of the method.
Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3**Error! Reference source not found.**.

***Limit of Quantification (LOQ)***

Definition: The limit of quantification is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than 1/10$^{th}$ of the value of the target concentration with an $RSD_r$ ≤25%. Target concentration should be intended as the threshold relevant for legislative requirements.
Example: For a 0.9% nominal value LOQ <0.09%.

***Limit of Detection (LOD)***

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single-laboratory validation.

Acceptance Criterion: LOD should be less than 1/20$^{th}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring ≤5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.
Example: For a 0.9% nominal value LOD <0.045%.

***Robustness***

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.
The adequacy of the robustness testing needs to be demonstrated on a method-by-method basis. For instance, for a real-time PCR method, the following factors and their origin/source shall be taken into account: different thermal cycler models, DNA polymerase, uracyl-n-glycosylase, magnesium chloride concentration, primer forward and reverse concentration, probe concentration, temperature profile, time profile, dNTP including dUTP concentrations.

Acceptance Criterion: The response of an assay with respect to these small changes shall not deviate more than ±30%.
Alternatively, robustness can be demonstrated through the application of formal robustness tests using factorial designs such as those published by Plackett Burman [1] or Yuden[2]

**Method Performance Requirements**

***Precision - Relative Reproducibility Standard Deviation (RSDR)***

Definition: The relative standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation $RSD_R$ should be <35% over the whole dynamic range. However, at concentrations <0.2% then $RSD_R$ values <50% are deemed acceptable.

***Trueness***

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within ±25% of the accepted reference value over the whole dynamic range.

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1) R.L. Plackett and J.P. Burman, "The Design of Optimum Multifactorial Experiments", Biometrika 33 (4), pp. 305-25, June 1946.

2) Statistical Manual of the AOAC, W.J. Youdens and E.H. Steiner, 1987.

# Event-specific Method for the Quantification of Soybean MON87769 Using Real-time PCR

## Protocol

**17 January 2012**

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**Institute for Health and Consumer Protection**
**Molecular Biology and Genomics Unit**

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# Content

# 1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR procedure for the determination of the relative content of soybean event MON87769 DNA to total soybean DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of soybean event MON87769 DNA, an 87-bp fragment of the integration region spanning the 3′ plant-to-insert junction in soybean MON87769 event is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxylfluorecein), as a reporter at its 5′ end, and TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3′ end.

For the relative quantification of soybean event MON87769, a soybean-specific reference system amplifies a 74-bp fragment of *Le1*, a soybean endogenous gene encoding lectin (GenBank: K00821.1), using specific primers and a *Le1* gene-specific probe labelled with FAM as a reporter dye at its 5′ end, and TAMRA as a quencher dye at its 3′ end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event MON87769 DNA in a test sample, Ct values for the MON87769 and the *Le1* are determined for the sample. Standard curves are then used to estimate the relative amount of soybean event MON87769 DNA to total soybean DNA.

# 2. Validation and performance characteristics

## 2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional soybean seeds.

The reproducibility and trueness of the method were assessed through an international collaborative ring trial using DNA samples at different GMO contents.

## 2.2 Collaborative trial

The method was validated by the EU-RL GMFF in an international collaborative study. The study was undertaken with twelve participating laboratories in July 2010.

Each participant received twenty blind samples containing soybean MON87769 genomic DNA at five GM contents, ranging from 0.1% to 9.0%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of event MON87769 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm.

## 2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is ≤ 0.04% in 200 ng of total soybean DNA. The relative LOD was not assessed in the collaborative study.

## 2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is ≤ 0.085% in 200 ng of total soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.09%.

## 2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome; the sequence is specific to soybean event MON87769 and thus imparts event-specificity to the method.

The specificity of both the event-specific and the soybean-specific assays was assessed by the method developer in real-time PCR against DNA extracted from plant materials containing the specific targets of maize GA21, NK603, MON810, MON863, MON88017, LY038, MON88034, MON87460 and conventional maize; canola RT73, RT200 and conventional canola; soybean 40-3-2, MON89788 and conventional soybean; wheat MON71800 and conventional wheat; cotton MON531, MON15985, MON1445, MON88913 and conventional cotton; conventional millet, lentils, sunflowers, peanuts (shelled), and quinoa.

According to the method developer, the MON87769 assay did not react with any of the plant materials tested, except the MON87769 positive control; the soybean-specific reference system reacted only with conventional soybean and with the soybean GM events tested.

The EU-RL GMFF assessed *in silico* the specificity of the event-specific assay by running similarity searches against the Central Core Sequence Information System (CCSIS). The CCSIS contains non-publicly available DNA sequences of GM events retrieved from applications for authorisation according to Regulation (EC) No 1829/2003 on GM Food and Feed. The sequence records are stored locally on a dedicated server integrated with common bioinformatics

applications (BLAST, ClustalW, EMBOSS package) for immediate bioinformatics analyses and are accessible with restricted user-admittance.

The results of the bioinformatics analysis showed that the forward primer of the event-specific assay has a 100% similarity with a number of patented sequences and other GM events. The results showed that this primer is similar to a sequence of *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA. However, no significant similarity for the reverse primer was found in the CCSIS.

# 3. Procedure

## 3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. "forward flow direction" during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- PCR reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- Equipment used should be sterilised prior to use and any residue of DNA should be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications; it must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps, unless specified otherwise, should be carried out at 0 – 4°C.
- In order to avoid repeated freeze/thaw cycles, aliquots should be prepared.

## 3.2 Real-time PCR for quantitative analysis of soybean event MON87769

### 3.2.1 General

The PCR set-up for the taxon-specific target sequence (*Le1*) and for the GMO (event MON87769) target sequence is carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended. The method is developed for a total volume of 50 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

### 3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% soybean MON87769 DNA in non-GM soybean DNA for a total of 200 ng of DNA (corresponding to approximately 85837 soybean genome copies with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA) [(1)]. The other four standards are prepared by serial dilution.

A calibration curve is produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. This can be done by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software. The copy number measured for each unknown sample DNA is obtained by interpolation from the standard curves.

### 3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. Keep thawed reagents on ice.
2. To prepare the amplification reaction mixtures add the following components (Tables 1 and 2) in two reaction tubes (one for the MON87769 assay and the other for the *Le1* assay) on ice in the order mentioned below (except DNA).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON87769 assay.

| Component | Final concentration | µL/reaction |
|---|---|---|
| TaqMan® Universal PCR Master Mix (2x) | 1x | 25 |
| MON87769 forward primer (10 µM) | 600 nM | 3 |
| MON87769 reverse primer (10 µM) | 600 nM | 3 |
| MON87769 probe (5 µM) | 200 nM | 2 |
| Nuclease free water | # | 13 |
| Template DNA (max 200 ng) | # | 4 |
| Total reaction volume: | | 50 |

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the soybean *Le1* reference assay.

| Component | Final concentration | µL/reaction |
|---|---|---|
| TaqMan® Universal PCR Master Mix (2x) | 1x | 25 |
| *Le1* primer 1 (10 µM) | 150 nM | 0.75 |
| *Le1* primer 2 (10 µM) | 150 nM | 0.75 |
| *Le1* probe (5 µM) | 50 nM | 0.5 |
| Nuclease free water | # | 19 |
| Template DNA (max 200 ng) | # | 4 |
| Total reaction volume: | | 50 |

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the soybean MON87769 and one for the *Le1* reaction mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of reaction mix (e.g. 46 µL x 3 = 138 µL reaction mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 4 µL x 3 = 12 µL DNA for three PCR repetitions). Vortex each tube for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
6. Spin down the tubes in a micro-centrifuge. Aliquot 50 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4°C to room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 3.

Table 3. Cycling program for MON87769/*Le1* assays

| Step | Stage | | T (°C) | Time (sec) | Acquisition | Cycles |
|---|---|---|---|---|---|---|
| 1 | UNG | | 50 | 120 | No | 1 |
| 2 | Initial denaturation | | 95 | 600 | No | 1 |
| 3 | Amplification | Denaturation | 95 | 15 | No | 45 |
| | | Annealing & Extension | 60 | 60 | Yes | |

## 3.3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curves of one assay (e.g. MON87769) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" (or "apply") button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).

c) Save the settings.

d) Repeat the procedure described in a) and b) on the amplification plots of the other assay (e.g. *Le1* system).

e) Save the settings and export all the data to a text file for further calculations.

## 3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct-values for each reaction.

The standard curves are generated both for the *Le1* and the MON87769 specific assays by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the DNA copy numbers in the unknown samples.

To obtain the percentage value of event MON87769 DNA in the unknown sample, the MON87769 copy number is divided by the copy number of the soybean reference gene (*Le1*) and multiplied by 100 (GM% = MON87769/*Le1* x 100).

# 4. Materials

## 4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

## 4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437.

## 4.3 Primers and Probes

| Name | Oligonucleotide DNA Sequence (5' to 3') | Length (nt) |
|---|---|---|
| MON 87769 target sequence | | |
| MON 87769 forward primer | 5' – CAT ACT CAT TgC TgA TCC ATg TAg ATT - 3' | 27 |
| MON 87769 reverse primer | 5' – gCA AgT TgC TCg TgA AgT TTT g - 3' | 22 |
| MON 87769 probe | 6-FAM 5' – CCC ggA CAT gAA gCC ATT TAC AAT TgA C - 3' TAMRA | 28 |
| Reference gene *Le1* target sequence | | |
| *Le1* primer 1 | 5' - CCA gCT TCg CCg CTT CCT TC - 3' | 20 |
| *Le1* primer 2 | 5' - gAA ggC AAg CCC ATC TgC AAg CC - 3' | 23 |
| *Le1* probe | 6-FAM 5' - CTT CAC CTT CTA TgC CCC TgA CAC -3' TAMRA | 24 |

FAM: 6-carboxyfluorescein; TAMRA: tetramethylrhodamine

# 5. References

1. Royal Botanic Garden, Kew. Plant DNA c-values database. http://data.kew.org/cvalues/

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Abstract

The European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF), established by Regulation (EC) No 1829/2003, in collaboration with the European Network of GMO Laboratories (ENGL), has carried out a collaborative study to assess the performance of a quantitative event-specific method to detect and quantify the MON87769 transformation event in soybean DNA (unique identifier MON-87769-7). The collaborative trial was conducted according to internationally accepted guidelines [1, 2].

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 “on genetically modified food and feed” and to Regulation (EC) No 641/2004 of 6 April 2004 “on detailed rules for the implementation of Regulation (EC) No 1829/2003”, Monsanto provided the detection method and the control samples (genomic DNA extracted from soybean seeds harbouring the MON87769 event and from conventional soybean seeds). The EU-RL GMFF prepared the validation samples (calibration samples and samples at unknown GM percentage [DNA/DNA]). The collaborative trial involved twelve laboratories from nine European countries.

The results of the international collaborative trial met the ENGL performance requirements. The method is therefore considered applicable to the control samples provided, in accordance with the requirements of Annex I-2.C.2 to Regulation (EC) No 641/2004.

The results of the collaborative study are made publicly available at http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm

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